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Synergistic Activities of Fluconazole and Voriconazole with Terbinafine against Four *Candida* Species Determined by Checkerboard, Time-Kill, and Etest Methods

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The in vitro activities of fluconazole or voriconazole plus terbinafine were evaluated against 20 *Candida* isolates by the checkerboard, time-kill, and Etest methods. Synergism (*C. albicans*, *C. glabrata*, and *C. tropicalis*) and indifference (*C. krusei*) were observed. Correlation among methods was good. The Etest is a suitable method to determine drug interactions.

The checkerboard and time-kill methods to determine in vitro interactions between drugs are time-consuming and cumbersome for use in clinical laboratories. In order to find a method that facilitates synergistic studies, our aim was dual: (i) to assess the in vitro activities of voriconazole (VRC) and fluconazole (FLC) combined with terbinafine (TRB) against four *Candida* spp. (resistant or susceptible to FLC and/or TRB) by the checkerboard and time-kill methods and (ii) to compare the results of these methods with those obtained by an Etest-agar dilution technique.

Twenty blood isolates (Table 1) were tested. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included for quality control.

Stock solutions of VRC, FLC (Pfizer, Barcelona, Spain), and TRB (Novartis, Barcelona, Spain) were prepared with the appropriate solvent (dimethyl sulfoxide for VRC and TRB and distilled water for FLC). The final concentrations were 0.002 to 2 μg/ml for VRC, 0.06 to 64 μg/ml for FLC, and 0.25 to 16 μg/ml for TRB. MICs of drugs alone or in combination were determined by the NCCLS M27-A2 method (12) and corresponded to the lowest concentration that showed prominent (≥50%) growth inhibition and by the Etest method as described below.

Drug interactions were assessed by the following three methods described below: broth microdilution checkerboard, time-kill, and Etest.

(i) Broth microdilution checkerboard. The broth microdilution checkerboard method was performed by using the fractional inhibitory concentration (FIC) index, which is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone. For computation of FIC indices, off-scale MICs were raised to the next highest MIC; synergistic and antagonistic FIC indices were defined as ≤0.5 and >4, respectively.

(ii) Time-kill studies. One isolate of each species was selected, and tests were conducted as previously described (RPMI 1640 medium, 10^5-CFU/ml inoculum, and 5-ml volume) (8). The drug concentrations tested alone were as follows: VRC, 16 and 1 μg/ml; FLC, 32 and 2 μg/ml; and TRB, 8 and 2 μg/ml. For the combinationsVRC/TRB and FLC/TRB, the drug concentrations were as follows: VRC/TRB, 16/2, 1/2, and 1/8 μg/ml; and FLC/TRB, 32/2, 32/8, 2/2, and 2/8 μg/ml. At 0, 3, 6, 24, and 48 h, aliquots were removed to determine the number of CFU per milliliter. Synergy was defined as a ≥2-$\log_{10}$ decrease in CFU per milliliter for a combination compared to the killing with the most active drug alone and an increase of ≥2 $\log_{10}$ as antagonism. Experiments were conducted in duplicate and on 2 separate days.

(iii) Etest studies. RPMI 1640 agar with 2% dextrose and 1, 2, and 8 μg of TRB per ml, prepared as described elsewhere (7), was used. For each strain, FLC or VRC Etest strips were applied to two agar plates, one with TRB (MIC of the combination) and another without it (azole MIC). Plates were inoculated following the manufacturer's instructions; MICs were obtained at 24 and 48 h. An azole MIC reduction of ≥3 dilutions in the presence of TRB was defined as synergy, and an increase of ≥3 dilutions was defined as antagonism.

MICs for the quality control strains were within the acceptable range (6, 12). Although 24-h Etest MICs were within 2 dilutions compared with those obtained by M27-A2 (48 h) for most isolates, the M27-A2 method detected resistance while Etest provided susceptible results for some *C. tropicalis* isolates (Tables 1 to 3); however, Etest ellipses had heavy trailing growth.

By checkerboard, the combination of both azoles and TRB was synergistic against four strains each of *C. albicans*, *C. glabrata*, and *C. tropicalis* (ΣFIC index, ≤0.5) (Table 1). Against *C. albicans*, MICs of FLC and VRC were 0.06 and 0.002 to 0.03 μg/ml, respectively, when combined with 0.25 to 0.5 μg of TRB per ml. Both azoles in combination with TRB inhibited the growth of *C. albicans*. Against *C. glabrata*, FLC MICs decreased to 1 to 4 μg/ml upon combination with 0.5 to 2 μg of TRB per ml. VRC MICs decreased 2 to 3 dilutions when

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TABLE 1. In vitro interaction between FLC and TRB and between VRC and TRB by the checkerboard method

Isolate	MIC (μg/ml)			Lowest ΣFIC for FLC/TRB (interpretation)[a]	MIC (μg/ml)			Lowest ΣFIC for VRC/TRB (interpretation)[a]
	FLC	TRB	FLC/TRB		VRC	TRB	VRC/TRB	
C. albicans								
EU-62	>64	16	0.06/0.25	0.016 (S)	>2	>16	0.002/0.5	0.016 (S)
EU-78	1	0.25	0.06/0.25	1.12 (I)	0.008	0.5	0.004/0.25	1.00 (I)
EU-80	>64	>16	0.06/0.5	0.016 (S)	>2	>16	0.002/0.5	0.016 (S)
EU-87	64	16	0.06/0.25	0.016 (S)	>2	>16	0.002/0.5	0.016 (S)
EU-170	2	>16	0.06/0.5	0.054 (S)	>2	8	0.03/0.5	0.07 (S)
C. glabrata								
EU-12	>64	>16	4/0.5	0.046 (S)	>2	>16	0.5/0.25	0.132 (S)
EU-38	16	>16	32/4	2.125 (I)	>2	>16	1/4	0.375 (S)
EU-68	8	>16	1/1	0.156 (S)	>2	>16	1/4	0.375 (S)
EU-151	32	>16	1/2	0.093 (S)	0.5	>16	0.5/0.25	1.007 (I)
EU-195	16	>16	4/2	0.312 (S)	>2	>16	0.25/2	0.125 (S)
C. tropicalis								
EU-43	>64	>16	1/0.5	0.023 (S)	0.12	>16	0.002/8	0.266 (S)
EU-240	>64	>16	16/2	0.187 (S)	>2	>16	0.25/2	0.125 (S)
EU-245	>64	>16	0.5/0.25	0.0117 (S)	1	>16	0.03/8	0.282 (S)
EU-255	>64	16	1/0.5	0.023 (S)	>2	>16	0.016/8	0.254 (S)
EU-264	1	>16	0.5/0.25	0.5078 (I)	0.03	>16	0.016/0.25	0.5078 (I)
C. krusei								
EU-123	64	>16	64/0.25	1.0078 (I)	0.5	>16	0.5/0.25	1.0078 (I)
CK-1	64	>16	64/0.25	1.0078 (I)	0.5	>16	0.25/0.25	0.5078 (I)
CK-2	>64	>16	64/16	1 (I)	1	>16	0.25/0.25	0.2578 (S)
CK-3	64	>16	64/0.25	1.0078 (I)	0.5	>16	0.25/0.25	0.5078 (I)
CK-4	64	>16	64/0.25	1.0078 (I)	0.5	>16	0.5/0.25	1.0078 (I)

[a] S, synergism; I, indifference.

combined with ≥0.5 μg of TRB per ml, and TRB MICs were reduced to 0.25 to 4 μg/ml in the presence of ≥0.25 μg of VRC per ml (Table 1). MICs of FLC for *C. tropicalis* were ≤1 μg/ml combined with TRB (≥0.25 μg/ml), except for one strain, for which the FLC MIC was 16 μg/ml in presence of 2 μg of TRB per ml. VRC MICs were ≤0.25 μg/ml when combined with ≥2 μg of TRB per ml. Against *C. krusei*, the interaction of both azoles with TRB was indifferent for four strains (ΣFIC

TABLE 2. Effect of TRB concentration on FLC activity as determined by the checkerboard and Etest methods

Strain	FLC MIC (μg/ml) with TRB concn of[a]:							
	0		1 μg/ml		2 μg/ml		8 μg/ml	
	M27-A2	Etest	Checkerboard	Etest	Checkerboard	Etest	Checkerboard	Etest
C. albicans								
EU-62	>64	64	<0.06–1*	0.016*	0.06–1*	0.06*	0.06–1*	0.12*
EU-78	1	0.25	<0.06	2*	0.06–1*	0.12*	0.06–1*	0.06*
EU-80	>64	>256	<0.06	0.5	0.06–1*	0.25*	0.06–1*	1.5*
EU-87	64	64	<0.06	0.5	0.06–1*	0.25*	0.06–1*	0.38*
EU-170	2	0.5	<0.06	0.75	0.06	0.25*	0.06–1*	0.38*
C. glabrata								
EU-12	>64	>256	4	ND[b]	4	1	1	0.5
EU-38	16	24	64	ND	64	32	32	16
EU-68	8	16	8	ND	8	8	0.5	8
EU-151	32	32	8	ND	1	8	1	8
EU-195	16	16	8	ND	4	16	4	2
C. tropicalis								
EU-43	>64	1	1	ND	1	1	0.5	1
EU-240	>64	12	32	ND	16	12	16	12
EU-245	>64	0.5	0.5	ND	0.5	0.38	0.12	0.38
EU-255	>64	0.75	1	ND	1	2	<0.06	2
EU-264	1	1	0.5	ND	0.5	1	0.5	1
C. krusei								
EU-123	64	64	64	ND	64	32	64	32
CK-1	64	64	64	ND	64	64	64	32
CK-2	>64	>64	>64	ND	>64	128	>64	64
CK-3	64	64	64	ND	64	48	64	48
CK-4	64	>256	64	ND	64	48	64	64

[a] Asterisks indicate the minimum drug concentration that produced 100% growth inhibition.
[b] ND, not determined.

TABLE 3. Effect of TRB concentration on VRC activity as determined by the checkerboard and Etest methods

Strain	VRC MIC (μg/ml) with TRB concn of:							
	0		1 μg/ml		2 μg/ml		8 μg/ml	
	M27-A2	Etest	Checkerboard	Etest	Checkerboard	Etest	Checkerboard	Etest
C. albicans								
EU-62	>2	>32	0.002	0.002	0.03*	0.004*	0.002*	0.008*
EU-78	0.008	0.008	0.002*	0.008*	0.002*	0.004*	0.002*	0.008*
EU-80	>2	>32	0.002–0.06*	0.002	0.002*	0.002*	0.002*	0.016*
EU-87	>2	>32	0.002*	0.016*	0.002*	0.016*	0.002*	0.012*
EU-170	>2	>32	0.016	0.016	0.03*	0.012*	0.002*	0.012*
C. glabrata								
EU-12	>2	>32	0.5	ND[b]	0.12	0.5	0.12	0.5
EU-38	>2	16	>2	ND	>2	0.5	1	0.5
EU-68	>2	8	2	ND	2	8	1	2
EU-151	0.5	2	2	ND	2	1	0.5	0.25
EU-195	>2	0.5	0.5	ND	0.25	0.38	0.12	0.12
C. tropicalis								
EU-43	0.12	0.06	2	ND	0.25	0.12	0.002	0.12
EU-240	>2	0.19	>2	ND	0.25	0.38	0.25	0.5
EU-245	1	0.06	0.12	ND	0.12	0.12	0.016	0.047
EU-255	>2	0.25	>2	ND	2	0.25	0.016	0.5
EU-264	0.03	0.12	0.016	ND	0.016	0.06	0.016	0.19*
C. krusei								
EU-123	0.5	0.25	0.5	ND	0.5	0.19*	0.5*	0.25*
CK-1	0.5	0.25	0.25	ND	0.25	0.25*	0.25*	0.12*
CK-2	1	0.5	0.5	ND	0.5	0.5*	0.5*	0.5*
CK-3	0.5	0.25	0.25	ND	0.25	0.25*	0.25*	0.25*
CK-4	0.5	0.25	0.5	ND	0.5	0.25*	0.5*	0.25*

[a] Asterisks indicate the minimum drug concentration that produced 100% growth inhibition.
[b] ND, not determined.

index, 0.5078 to 1.01), while the combination VRC/TRB was synergistic (ΣFIC index = 0.2578) for one isolate (Table 1).

By the time-kill method, the combination of both azoles with TRB was indifferent: none of the killing curves showed more than a 1.5-log decrease in killing in either of the two independent tests made (data not shown).

Tables 2 and 3 depict the interaction of azoles with TRB by Etest. In general, Etest synergistic results were in agreement with those obtained by the checkerboard method with the same concentrations of TRB.

Our results by the checkerboard method confirm previous reports (4, 5, 14, 19) and extend them to *C. tropicalis* and *C. krusei*. We found that the combination of FLC or VRC with TRB was synergistic for *C. albicans*, *C. glabrata* (strain dependent), and *C. tropicalis* and indifferent for *C. krusei* by the checkerboard and Etest methods. However, by the time-kill method, the interaction was indifferent. The lowest FIC indices for the combination of both azoles and TRB were obtained at achievable concentrations in serum of 3.6 μg/ml for TRB (3, 11, 18), 30 μg/ml for FLC (9, 17, 18), and up to 5.2 μg/ml for VRC (2, 10, 16). The three methods showed good correlation for most of the species, although by the checkerboard method, the synergistic effect was stronger. The lack of agreement between the checkerboard and Etest methods for *C. tropicalis* could be due to the heavy trailing growth observed at 48 h by the microdilution method; agreement was good with 24-h MICs by the latter method. To our knowledge, there are no other reports describing the same drug combinations, species, and methods. It is interesting that when the TRB concentration that produced the interaction with the azole was reached, a further increase did not decrease the azole MIC. This was demonstrated by the three methods.

By using available Etest strips and incorporating subinhibitory concentrations of TRB into the RPMI agar, we were able to determine interactions between these agents. These results were similar to those obtained by the most frequently used checkerboard method (Tables 2 and 3). The Etest could be a suitable method in clinical laboratories, because both RPMI agar and Etest strips are commercially available for established agents. In addition, the incorporation of the new agent into the agar plate can be made by flooding the agar plate with the appropriate drug concentrations as recommended in the NCCLS M44-P document (13). Our methodology could also be useful to study the interaction of antifungal agents with other substances (antineoplasic, anti-inflammatory, immunosuppressive drugs, etc.) (1, 15). Further studies are needed to determine reliability of these methods and the correlation of in vitro and in vivo results.

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